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Title Of Invention

Cloned Genome Of Infectious
Hepatitis C Virus of Genotype 2a And Uses Thereof

Field Of Invention

The present invention relates to molecular approaches to the production of nucleic acid sequence which comprises the genome of infectious hepatitis C virus. In particular, the invention provides a nucleic acid sequence which comprises the genome of an infectious hepatitis C virus of genotype 2a. The invention therefore relates to the use of the nucleic acid sequence and polypeptides encoded by all or part of the sequence in the development of vaccines and diagnostic assays for HCV and in the development of screening assays for the identification of antiviral agents for HCV.

Background Of Invention

Hepatitis C virus (HCV) has a positive-sense single-strand RNA genome and is a member of the genus *Hepacivirus* within the *Flaviviridae* family of viruses (Rice, 1996). As for all positive-stranded RNA viruses, the genome of HCV functions as mRNA from which all viral proteins necessary for propagation are translated.

The viral genome of HCV is approximately 9600 nucleotides (nts) in length and consists of a highly conserved 5' untranslated region (UTR), a single long open reading frame (ORF) of approximately 9,000 nts and a complex 3' UTR. The 5' UTR contains an internal ribosomal entry site (Tsukiyama-Kohara et al., 1992;

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0 Honda et al., 1996). The 3' UTR consists of a short
variable region, a polypyrimidine tract of variable
length and, at the 3' end, a highly conserved region of
approximately 100 nucleotides (Kolykhalov et al., 1996;
5 Tanaka et al., 1995; Tanaka et al., 1996; Yamada et al.,
1996). The last 46 nucleotides of this conserved region
were predicted to form a stable stem-loop structure
thought to be critical for viral replication (Blight and
10 Rice, 1997; Ito and Lai, 1997; Tsuchihara et al., 1997).
The ORF encodes a large polypeptide precursor that is
cleaved into at least 10 proteins by host and viral
proteinases (Rice, 1996). The predicted envelope
proteins contain several conserved N-linked
15 glycosylation sites and cysteine residues (Okamoto et
al., 1992a). The NS3 gene encodes a serine protease and
an RNA helicase and the NS5B gene encodes an RNA-
dependent RNA polymerase.

20 A remarkable characteristic of HCV is its
genetic heterogeneity, which is manifested throughout
the genome (Bukh et al., 1995). The most heterogeneous
regions of the genome are found in the envelope genes,
in particular the hypervariable region 1 (HVR1) at the
25 N-terminus of E2 (Hijikata et al., 1991; Weiner et al.,
1991). HCV circulates as a quasispecies of closely
related genomes in an infected individual. Globally,
six major HCV genotypes (genotypes 1-6) and multiple
30 subtypes (a, b, c, etc.) have been identified (Bukh et
al., 1993; Simmonds et al., 1993).

The nucleotide and deduced amino acid
sequences among isolates within a quasispecies generally
differ by < 2%, whereas those between isolates of
35 different genotypes vary by as much as 35%. Genotypes

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1, 2 and 3 are found worldwide and constitute more than 90% of the HCV infections in North and South America, Europe, Russia, China, Japan and Australia (Forns and Bukh, 1998). Throughout these regions genotype 1 accounts for the majority of HCV infections but genotypes 2 and 3 each account for 5-15%.

At present, more than 80% of individuals infected with HCV become chronically infected and these chronically infected individuals have a relatively high risk of developing chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Hoofnagle, 1997). The only effective therapy for chronic hepatitis C, interferon (IFN), alone or in combination with ribavirin, induces a sustained response in less than 50% of treated patients (Davis et al., 1998; McHutchinson et al., 1998). Consequently, HCV is currently the most common cause of end stage liver failure and the reason for about 30% of liver transplants performed in the U.S. (Hoofnagle, 1997). In addition, a number of recent studies suggested that the severity of liver disease and the outcome of therapy may be genotype-dependent (reviewed in Bukh et al., 1997). In particular, these studies suggested that infection with HCV genotype 1b was associated with more severe liver disease (Brecht, 1997) and a poorer response to IFN therapy (Fried and Hoofnagle, 1995). As a result of the inability to develop a universally effective therapy against HCV infection, it is estimated that there are still more than 25,000 new infections yearly in the U.S. (Alter 1997) Moreover, since there is no vaccine for HCV, HCV remains a serious public health problem.

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Despite the intense interest in the development of vaccines and therapies for HCV, progress has been hindered by the absence of a useful cell culture system and the lack of any small animal model for laboratory study. For example, while replication of HCV in several cell lines has been reported, such observations have turned out not to be highly reproducible. In addition, the chimpanzee is the only animal model, other than man, for this disease. Consequently, HCV has been studied only by using clinical materials obtained from patients or experimentally infected chimpanzees, an animal model whose availability is very limited.

However, several researchers have recently reported the construction of infectious cDNA clones of HCV, the identification of which would permit a more effective search for susceptible cell lines and facilitate molecular analysis of the viral genes and their function. For example, Yoo et al., and Dash et al., (1997) (1995) reported that RNA transcripts from cDNA clones of HCV-1 (genotype 1a) and HCV-N (genotype 1b), respectively, resulted in viral replication after transfection into human hepatoma cell lines. Unfortunately, the viability of these clones was not tested in vivo and concerns were raised about the infectivity of these cDNA clones in vitro (Fausto, 1997). In addition, both clones did not contain the terminal 98 conserved nucleotides at the very 3' end of the UTR.

Kolykhalov et al., (1997) and Yanagi et al. (1997, 1998) reported the derivation from HCV strains H77 (genotype 1a) and HC-J4 (genotype 1b) of cDNA clones

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of HCV that are infectious for chimpanzees. However, while these infectious clones will aid in studying HCV replication and pathogenesis and will provide an important tool for development of in vitro replication and propagation systems, it is important to have infectious clones of more than one genotype, given the extensive genetic heterogeneity of HCV and the potential impact of such heterogeneity on the development of effective therapies and vaccines for HCV.

In addition, synthetic chimeric viruses can be used to map the functional regions of viruses with different phenotypes. In flaviviruses and pestiviruses, infectious chimeric viruses have been successfully engineered to express different functional units of related viruses (Bray and Lai, 1991; Pletnev et al., 1992, 1998; Vassilev et al., 1997) and in some cases it has been possible to make chimeras between non-related or distantly related viruses. For instance, the IRES element of poliovirus or bovine viral diarrhea virus has been replaced with IRES sequences from HCV (Frolov et al., 1998; Lu and Wimmer, 1996; Zhao et al., 1999). Recently, the construction of an infectious chimera of two closely related HCV subtypes has been reported. The chimera contained the complete ORF of a genotype 1b strain but had the 5' and 3' termini of a genotype 1a strain (Yanagi et al., 1998).

It is important to determine whether chimeras constructed from more divergent HCV strains are infectious because such chimeras could be used to define the functions of viral units and to dissect the immune response.

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Summary Of The Invention

The present invention relates to nucleic acid sequence which comprises the genome of infectious hepatitis C virus and in particular, nucleic acid sequence which comprises the genome of infectious hepatitis C virus of genotype 2a. It is therefore an object of the invention to provide nucleic acid sequence which encodes infectious hepatitis C virus. Such nucleic acid sequence is referred to throughout the application as "infectious nucleic acid sequence".

For the purposes of this application, nucleic acid sequence refers to RNA, DNA, cDNA or any variant thereof capable of directing host organism synthesis of a hepatitis C virus polypeptide. It is understood that nucleic acid sequence encompasses nucleic acid sequences, which due to degeneracy, encode the same polypeptide sequence as the nucleic acid sequences described herein.

The invention also relates to the use of the infectious nucleic acid sequences to produce chimeric genomes consisting of portions of the open reading frames of nucleic acid sequences of other genotypes (including, but not limited to, genotypes 1, 2, 3, 4, 5 and 6) and subtypes (including, but not limited to, subtypes 1a, 1b, 2a, 2b, 2c, 3a, 4a-4f, 5a and 6a) of HCV. For example, infectious nucleic acid sequence of the 2a strain HC-J6, described herein can be used to produce chimeras with sequences from the genomes of other strains of HCV from different genotypes or subtypes. Nucleic acid sequences which comprise sequences from two or more HCV genotypes or subtypes are designated "chimeric nucleic acid sequences".

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The invention further relates to mutations of the infectious nucleic acid sequence of the invention where mutation includes, but is not limited to, point mutations, deletions and insertions. In one embodiment, a gene or fragment thereof can be deleted to determine the effect of the deleted gene or genes on the properties of the encoded virus such as its virulence and its ability to replicate. In an alternative embodiment, a mutation may be introduced into the infectious nucleic acid sequences to examine the effect of the mutation on the properties of the virus.

The invention also relates to the introduction of mutations or deletions into the infectious nucleic acid sequence in order to produce an attenuated hepatitis C virus suitable for vaccine development.

The invention further relates to the use of the infectious nucleic acid sequence to produce attenuated viruses via passage in vitro or in vivo of the viruses produced by transfection of a host cell with the infectious nucleic acid sequence.

The present invention also relates to the use of the nucleic acid sequence of the invention or fragments thereof in the production of polypeptides where "nucleic acid sequence of the invention" refers to infectious nucleic acid sequence, mutations of infectious nucleic acid sequence, chimeric nucleic acid sequence and sequences which comprise the genome of attenuated viruses produced from the infectious nucleic acid sequence of the invention. In one embodiment, said polypeptide or polypeptides are fully or partially purified from hepatitis C virus produced by cells transfected with nucleic acid sequence of the invention.

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° In another embodiment, the polypeptide or polypeptides are produced recombinantly from a fragment of the nucleic acid sequences of the invention. In yet another embodiment, the polypeptides are chemically synthesized.

5 The polypeptides of the invention, especially structural polypeptides, can serve as immunogens in the development of vaccines or as antigens in the development of diagnostic assays for detecting the presence of HCV in biological samples.

10 The invention therefore also relates to vaccines for use in immunizing mammals especially humans against hepatitis C. In one embodiment, the vaccine comprises one or more polypeptides made from the nucleic acid sequence of the invention or fragment thereof. In
15 a second embodiment, the vaccine comprises a hepatitis C virus produced by transfection of host cells with the nucleic acid sequences of the invention.

20 The present invention therefore relates to methods for preventing hepatitis C in a mammal. In one embodiment the method comprises administering to a mammal a polypeptide or polypeptides encoded by the nucleic acid sequence of the invention in an amount
25 effective to induce protective immunity to hepatitis C. In another embodiment, the method of prevention comprises administering to a mammal a hepatitis C virus of the invention in an amount effective to induce
30 protective immunity against hepatitis C.

35 In yet another embodiment, the method of protection comprises administering to a mammal the nucleic acid sequence of the invention or a fragment thereof in an amount effective to induce protective immunity against hepatitis C.

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The invention also relates to hepatitis C viruses produced by host cells transfected with the nucleic acid sequence of the present invention.

5 The invention therefore also provides pharmaceutical compositions comprising the nucleic acid sequence of the invention and/or the encoded hepatitis C viruses. The invention further provides pharmaceutical compositions comprising polypeptides encoded by the
10 nucleic acid sequence of the invention or fragments thereof. The pharmaceutical compositions of the invention may be used prophylactically or therapeutically.

15 The invention also relates to antibodies to the hepatitis C virus of the invention or their encoded polypeptides and to pharmaceutical compositions comprising these antibodies.

20 The invention also relates to the use of the nucleic acid sequences of the invention to identify cell lines capable of supporting the replication of HCV in vitro.

25 The invention further relates to the use of the nucleic acid sequences of the invention or their encoded viral enzymes (e.g. NS3 serine protease, NS3 helicase, NS5B RNA polymerase) to develop screening assays to identify antiviral agents for HCV.

Brief Description Of Figures

30 Figure 1 shows the amplification and cloning of hepatitis C virus genotype 2a (strain HC-J6_{CH}). The nucleotide positions correspond to the sequence of PJ6CF, a full length cDNA clone of hepatitis C virus,
35 genotype 2a, strain HC-J6_{CH}. Products from polymerase

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chain reaction are also shown. The names of the clones obtained from these products are indicated (number of clones sequenced are shown in parenthesis). The composition of the full-length cDNA clone is shown at the bottom. The restriction enzymes used for cloning are indicated. An *Xba*I site in HC-J6_{CH} was eliminated by a silent substitution at position 5494.

Figure 2 shows tree analysis of clones amplified from an infectious acute phase plasma pool generated in a chimpanzee inoculated with human plasma containing strain HC-J6 (Okamoto *et al.*, 1991) as well as a tree of the predicted polyprotein sequence of HC-J6_{CH} and the infectious HC-J6_{CH} cDNA clone (pJ6CF). The nucleotide positions with deletions or insertions were stripped in the analysis of the clones. Multiple sequence alignments and tree analyses were performed with GeneWorks (Oxford Molecular Group) (Bukh *et al.*, 1995). Genotype designations are indicated. Other sequences included in the analysis are HC-J8 (Okamoto *et al.*, 1992), genotype 1a infectious clone BEBE1 (Nakao *et al.*, 1996), H77C (Yanagi *et al.*, 1997); genotype 1b infectious clone J4L6S (Yanagi *et al.*, 1998). The scale in each tree indicates the calculated genetic distance.

Figure 3 shows the alignment of the hypervariable region 1 sequences from 8 J6S clones of strain HC-J6_{CH}. HC-J6_{CH} represents the consensus amino acid sequence of the infectious plasma pool from an experimentally infected chimpanzee. HC-J6 is the published amino acid sequence of the original inoculum (Okamoto *et al.*, 1991).

Figure 4 shows the construction of four intertypic chimeric cDNA clones. White boxes are

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sequences derived from genotype 2a clone pJ6CF, and
black boxes are sequences derived from genotype 1a clone
pCV-H77C (Yanagi et al., 1997). An *NdeI* site (mutation
at position 9158 of pCV-H77C) was eliminated and an
artificial *NdeI* site (mutation at position 2765 of
pCV-H77C) was created by site-directed mutagenesis;
silent mutations are underlined.

Figures 5A and 5B show the alignment of the
nucleotide sequences of the 5' (Fig. 5A) and 3' UTRs
(Fig. 5B) and the amino acid sequences of E2/p7/NS2
junctions (Fig. 5B) in the intertypic 1a, 2a chimeric
cDNA clones. In the 5' UTR alignment, the first 39 nts
of core believed to be important for the IRES function
were included (Lemon and Honda, 1997). Top line: the
sequence of the infectious genotype 1a clone pCV-H77C
(Yanagi et al., 1997). Bottom line: the sequence of the
infectious genotype 2a clone pJ6CF. Dot: identity with
the sequence of H77C. Capital letter: different from the
sequence of H77C. Dash: deletion. Bold face: initiation
or stop codon of the ORF. Underlined: *AgeI* cleavage
site. Arrow: putative sites in the HCV polyprotein
cleaved by host signal peptidases. Numbering
corresponds to the sequence of pCV-H77C.

Figures 6A-6F show the nucleotide sequence of
the infectious hepatitis C virus clone of genotype 1a
strain H77C and Figures 6G-6H show the amino acid
sequence encoded by the clone.

Figures 7A-7F show the nucleotide sequence of
the infectious hepatitis C virus clone of genotype 1b
strain HC-J4 and Figures 7G-H show the amino acid
sequence encoded by the clone.

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DESCRIPTION OF THE INVENTION

The present invention relates to nucleic acid sequence which comprises the genome of an infectious hepatitis C virus. More specifically, the invention relates to nucleic acid sequence which encodes infectious hepatitis C virus of strain HC-J6_{CH}, genotype 2a. The infectious nucleic acid sequence of the invention is shown in SEQ ID NO:1 and is contained in a plasmid construct deposited with the American Type Culture Collection (ATCC) on May 28, 1999 and having ATCC accession number PTA-153.

The invention also relates to "chimeric nucleic acid sequences" where the chimeric nucleic acid sequences consist of open-reading frame sequences and/or 5' and/or 3' untranslated sequences taken from nucleic acid sequences of hepatitis C viruses of different genotypes or subtypes.

In one embodiment, the chimeric nucleic acid sequence consists of sequence from the genome of infectious HCV of genotype 2a which encodes structural polypeptides and sequence from the genome of a HCV of a different genotype or subtype which encodes nonstructural polypeptides.

Alternatively, the nonstructural region of infectious HCV of genotype 2a and structural region of a HCV of a different genotype or subtype may be combined. This will result in a chimeric nucleic acid sequence consisting of sequence from the genome of infectious HCV of genotype 2a which encodes nonstructural polypeptides and sequence from the genome of a HCV of a another genotype or subtype which encodes structural polypeptides.

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Preferably, the nucleic acid sequence from the genome of the infectious HCV clone of genotype 1a (deposited with the ATCC on June 2, 1999 ; Figures 6A-6F), or the nucleic acid sequence from the genome of the infectious HCV clone of genotype 1b (ATCC accession number 209596; Figures 7A-7F) is used to construct the chimeric nucleic acid sequence with the HCV of genotype 2a of the invention.

It is believed that the construction of such chimeric nucleic acid sequences will be of importance in studying the growth and virulence properties of hepatitis C virus and in the production of candidate hepatitis C virus vaccines suitable to confer protection against multiple genotypes of HCV. For example, one might produce a "multivalent" vaccine by putting epitopes from several genotypes or subtypes into one clone. Alternatively one might replace just a single gene from an infectious sequence with the corresponding gene from the genomic sequence of a strain from another genotype or subtype or create a chimeric gene which contains portions of a gene from two genotypes or subtypes. Examples of genes which could be replaced or which could be made chimeric, include, but are not limited to, the E1, E2 and NS4 genes.

The invention further relates to mutations of the infectious nucleic acid sequences where "mutations" include, but are not limited to, point mutations, deletions and insertions. Of course, one of ordinary skill in the art would recognize that the size of the insertions would be limited by the ability of the resultant nucleic acid sequence to be properly packaged within the virion. Such mutations could be produced by

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o techniques known to those of skill in the art such as site-directed mutagenesis, fusion PCR, and restriction digestion followed by religation.

5 In one embodiment, mutagenesis might be undertaken to determine sequences that are important for viral properties such as replication or virulence. For example, one may introduce a mutation into the infectious nucleic acid sequence which eliminates the cleavage site between the NS4A and NS4B polypeptides to
10 examine the effects on viral replication and processing of the polypeptide.

Alternatively, one may delete all or part of a gene or of the 5' or 3' nontranslated region contained in
15 an infectious nucleic acid sequence and then transfect a host cell (animal or cell culture) with the mutated sequence and measure viral replication in the host by methods known in the art such as RT-PCR. Preferred
20 genes include, but are not limited to, the P7, NS4B and NS5A genes. Of course, those of ordinary skill in the art will understand that deletion of part of a gene, preferably the central portion of the gene, may be preferable to deletion of the entire gene in order to
25 conserve the cleavage site boundaries which exist between proteins in the HCV polyprotein and which are necessary for proper processing of the polyprotein.

In the alternative, if the transfection is
30 into a host animal such as a chimpanzee, one can monitor the virulence phenotype of the virus produced by transfection of the mutated infectious nucleic acid sequence by methods known in the art such as measurement of liver enzyme levels (alanine aminotransferase (ALT)
35 or isocitrate dehydrogenase (ICD)) or by histopathology

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° of liver biopsies. Thus, mutations of the infectious nucleic acid sequences may be useful in the production of attenuated HCV strains suitable for vaccine use.

5 The invention also relates to the use of the infectious nucleic acid sequence of the present invention to produce attenuated viral strains via passage in vitro or in vivo of the virus produced by transfection with the infectious nucleic acid sequence.

10 The present invention therefore relates to the use of the nucleic acid sequence of the invention to identify cell lines capable of supporting the replication of HCV.

15 In particular, it is contemplated that the mutations of the infectious nucleic acid sequence of the invention and the production of chimeric sequences as discussed above may be useful in identifying sequences critical for cell culture adaptation of HCV and hence, may be useful in identifying cell lines capable of
20 supporting HCV replication.

25 Transfection of tissue culture cells with the nucleic acid sequences of the invention may be done by methods of transfection known in the art such as electroporation, precipitation with DEAE-Dextran or calcium phosphate or liposomes.

30 In one such embodiment, the method comprises the growing of animal cells, especially human cells, in vitro and transfecting the cells with the nucleic acid of the invention, then determining if the cells show indicia of HCV infection. Such indicia include the detection of viral antigens in the cell, for example, by immunofluorescence procedures well known in the art; the
35 detection of viral polypeptides by Western blotting

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° using antibodies specific therefor; and the detection of newly transcribed viral RNA within the cells via methods such as RT-PCR. The presence of live, infectious virus particles following such tests may also be shown by
5 injection of cell culture medium or cell lysates into healthy, susceptible animals, with subsequent exhibition of the signs and symptoms of HCV infection.

Suitable cells or cell lines for culturing HCV include, but are not limited to, lymphocyte and
10 hepatocyte cell lines known in the art.

Alternatively, primary hepatocytes can be cultured, and then infected with HCV; or, the hepatocyte cultures could be derived from the livers of infected
15 chimpanzees. In addition, various immortalization methods known to those of ordinary skill in the art can be used to obtain cell lines derived from hepatocyte cultures. For example, primary hepatocyte cultures may be fused to a variety of cells to maintain stability.
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The present invention further relates to the in vitro and in vivo production of hepatitis C viruses from the nucleic acid sequences of the invention.

In one embodiment, the sequences of the
25 invention can be inserted into an expression vector that functions in eukaryotic cells. Eukaryotic expression vectors suitable for producing high efficiency gene transfer in vivo are well known to those of ordinary skill in the art and include, but are not limited to,
30 plasmids, vaccinia viruses, retroviruses, adenoviruses and adeno-associated viruses.

In another embodiment, the sequences contained in the recombinant expression vector can be transcribed
35 in vitro by methods known to those of ordinary skill in

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the art in order to produce RNA transcripts which encode the hepatitis C viruses of the invention. The hepatitis C viruses of the invention may then be produced by transfecting cells by methods known to those of ordinary skill in the art with either the in vitro transcription mixture containing the RNA transcripts or with the recombinant expression vectors containing the nucleic acid sequences described herein.

The hepatitis C viruses produced from the sequences of the invention may be purified or partially purified from the transfected cells by methods known to those of ordinary skill in the art. In a preferred embodiment, the viruses are partially purified prior to their use as immunogens in the pharmaceutical compositions and vaccines of the present invention.

The present invention therefore relates to the use of the hepatitis C viruses produced from the nucleic acid sequences of the invention as immunogens in live or killed (e.g., formalin inactivated) vaccines to prevent hepatitis C in a mammal.

In an alternative embodiment, the immunogen of the present invention may be an infectious nucleic acid sequence, a chimeric nucleic acid sequence, or a mutated infectious nucleic acid sequence which encodes a hepatitis C virus. Where the sequence is a cDNA sequence, the cDNAs and their RNA transcripts may be used to transfect a mammal by direct injection into the liver tissue of the mammal as described in the Examples.

Alternatively, direct gene transfer may be accomplished via administration of a eukaryotic expression vector containing a nucleic acid sequence of the invention.

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In yet another embodiment, the immunogen may be a polypeptide encoded by the nucleic acid sequences of the invention. The present invention therefore also relates to polypeptides produced from the nucleic acid sequences of the invention or fragments thereof. In one
5 embodiment, polypeptides of the present invention can be recombinantly produced by synthesis from the nucleic acid sequences of the invention or isolated fragments thereof, and purified, or partially purified, from
10 transfected cells using methods already known in the art. In an alternative embodiment, the polypeptides may be purified or partially purified from viral particles produced via transfection of a host cell with the
15 nucleic acid sequences of the invention. Such polypeptides might, for example, include either capsid or envelope polypeptides prepared from the sequences of the present invention.

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When used as immunogens, the nucleic acid sequences of the invention, or the polypeptides or viruses produced therefrom, are preferably partially purified prior to use as immunogens in pharmaceutical compositions and vaccines of the present invention.

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When used as a vaccine, the sequences and the polypeptide and virus products thereof, can be administered alone or in a suitable diluent, including, but not limited to, water, saline, or some type of
30 buffered medium. The vaccine according to the present invention may be administered to an animal, especially a mammal, and most especially a human, by a variety of routes, including, but not limited to, intradermally, intramuscularly, subcutaneously, or in any combination
35 thereof.

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Suitable amounts of material to administer for prophylactic and therapeutic purposes will vary depending on the route selected and the immunogen (nucleic acid, virus, polypeptide) administered. One skilled in the art will appreciate that the amounts to be administered for any particular treatment protocol can be readily determined without undue experimentation. The vaccines of the present invention may be administered once or periodically until a suitable titer of anti-HCV antibodies appear in the blood. For an immunogen consisting of a nucleic acid sequence, a suitable amount of nucleic acid sequence to be used for prophylactic purposes might be expected to fall in the range of from about 100 μ g to about 5 mg and most preferably in the range of from about 500 μ g to about 2mg. For a polypeptide, a suitable amount to use for prophylactic purposes is preferably 100 ng to 100 μ g and for a virus 10^2 to 10^6 infectious doses. Such administration will, of course, occur prior to any sign of HCV infection.

A vaccine of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. An inert carrier is preferably used, such as saline or phosphate-buffered saline, or any such carrier in which the HCV of the present invention can be suitably suspended. The vaccines may be in the form of single dose preparations or in multi-dose flasks which can be utilized for mass-vaccination programs of both animals and humans. For purposes of using the vaccines of the present invention reference is made to Remington's

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Pharmaceutical Sciences, Mack Publishing Co., Easton,
Pa., Osol (Ed.) (1980); and New Trends and Developments
in Vaccines, Voller et al. (Eds.), University Park
Press, Baltimore, Md. (1978), both of which provide much
5 useful information for preparing and using vaccines. Of
course, the polypeptides of the present invention, when
used as vaccines, can include, as part of the
composition or emulsion, a suitable adjuvant, such as
10 alum (or aluminum hydroxide) when humans are to be
vaccinated, to further stimulate production of
antibodies by immune cells. When nucleic acids, viruses
or polypeptides are used for vaccination purposes, other
specific adjuvants such as CpG motifs (Krieg, A.K. et
15 al. (1995) and (1996)), may prove useful.

When the nucleic acids, viruses and
polypeptides of the present invention are used as
vaccines or inocula, they will normally exist as
physically discrete units suitable as a unitary dosage
20 for animals, especially mammals, and most especially
humans, wherein each unit will contain a predetermined
quantity of active material calculated to produce the
desired immunogenic effect in association with the
25 required diluent. The dose of said vaccine or inoculum
according to the present invention is administered at
least once. In order to increase the antibody level, a
second or booster dose may be administered at some time
after the initial dose. The need for, and timing of,
30 such booster dose will, of course, be determined within
the sound judgment of the administrator of such vaccine
or inoculum and according to sound principles well known
in the art. For example, such booster dose could
35 reasonably be expected to be advantageous at some time

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° between about 2 weeks to about 6 months following the initial vaccination. Subsequent doses may be administered as indicated.

5 The nucleic acid sequences, viruses and polypeptides of the present invention can also be administered for purposes of therapy, where a mammal, especially a primate, and most especially a human, is already infected, as shown by well known diagnostic measures. When the nucleic acid sequences, viruses or
10 polypeptides of the present invention are used for such therapeutic purposes, much of the same criteria will apply as when it is used as a vaccine, except that inoculation will occur post-infection. Thus, when the
15 nucleic acid sequences, viruses or polypeptides of the present invention are used as therapeutic agents in the treatment of infection, the therapeutic agent comprises a pharmaceutical composition containing a sufficient
20 amount of said nucleic acid sequences, viruses or polypeptides so as to elicit a therapeutically effective response in the organism to be treated. Of course, the amount of pharmaceutical composition to be administered will, as for vaccines, vary depending on the immunogen
25 contained therein (nucleic acid, polypeptide, virus) and on the route of administration.

The therapeutic agent according to the present invention can thus be administered by subcutaneous,
30 intramuscular or intradermal routes. One skilled in the art will certainly appreciate that the amounts to be administered for any particular treatment protocol can be readily determined without undue experimentation. Of course, the actual amounts will vary depending on the
35 route of administration as well as the sex, age, and

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° clinical status of the subject which, in the case of human patients, is to be determined with the sound judgment of the clinician.

The therapeutic agent of the present invention can be employed in such forms as capsules, liquid solutions, suspensions or elixirs, or sterile liquid forms such as solutions or suspensions. An inert carrier is preferably used, such as saline, phosphate-buffered saline, or any such carrier in which the HCV of the present invention can be suitably suspended. The therapeutic agents may be in the form of single dose preparations or in the multi-dose flasks which can be utilized for mass-treatment programs of both animals and humans. Of course, when the nucleic acid sequences, viruses or polypeptides of the present invention are used as therapeutic agents they may be administered as a single dose or as a series of doses, depending on the situation as determined by the person conducting the treatment.

The nucleic acids, polypeptides and viruses of the present invention can also be utilized in the production of antibodies against HCV. The term "antibody" is herein used to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules. Examples of antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and portions of an immunoglobulin molecule, including those portions known in the art as Fab, F(ab')₂ and F(v) as well as chimeric antibody molecules.

Thus, the polypeptides, viruses and nucleic acid sequences of the present invention can be used in

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° the generation of antibodies that immunoreact (i.e., specific binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining site such as a whole antibody molecule or an active portion thereof) with antigenic determinants on the surface of hepatitis C virus particles.

5 The present invention therefore also relates to antibodies produced following immunization with the nucleic acid sequences, viruses or polypeptides of the present invention. These antibodies are typically produced by immunizing a mammal with an immunogen or vaccine to induce antibody molecules having

10 immunospecificity for polypeptides or viruses produced in response to infection with the nucleic acid sequences of the present invention. When used in generating such antibodies, the nucleic acid sequences, viruses, or polypeptides of the present invention may be linked to

15 some type of carrier molecule. The resulting antibody molecules are then collected from said mammal. Antibodies produced according to the present invention have the unique advantage of being generated in response

20 to authentic, functional polypeptides produced according to the actual cloned HCV genome.

The antibody molecules of the present invention may be polyclonal or monoclonal. Monoclonal antibodies are readily produced by methods well known in

25 the art. Portions of immunoglobulin molecules, such as Fabs, as well as chimeric antibodies, may also be produced by methods well known to those of ordinary skill in the art of generating such antibodies.

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The antibodies according to the present invention may also be contained in blood, plasma, serum, hybridoma supernatants, and the like. Alternatively, the antibody of the present invention is isolated to the extent desired by well known techniques such as, for example, using DEAE Sephadex. The antibodies produced according to the present invention may be further purified so as to obtain specific classes or subclasses of antibody such as IgM, IgG, IgA, and the like. Antibodies of the IgG class are preferred for purposes of passive protection.

The antibodies of the present invention are useful in the prevention and treatment of diseases caused by hepatitis C virus in animals, especially mammals, and most especially humans.

In providing the antibodies of the present invention to a recipient mammal, preferably a human, the dosage of administered antibodies will vary depending on such factors as the mammal's age, weight, height, sex, general medical condition, previous medical history, and the like.

In general, it will be advantageous to provide the recipient mammal with a dosage of antibodies in the range of from about 1 mg/kg body weight to about 10 mg/kg body weight of the mammal, although a lower or higher dose may be administered if found desirable. Such antibodies will normally be administered by intravenous or intramuscular route as an inoculum. The antibodies of the present invention are intended to be provided to the recipient subject in an amount sufficient to prevent, lessen or attenuate the severity, extent or duration of any existing infection.

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The antibodies prepared by use of the nucleic acid sequences, viruses or polypeptides of the present invention are also highly useful for diagnostic purposes. For example, the antibodies can be used as in
5 vitro diagnostic agents to test for the presence of HCV in biological samples taken from animals, especially humans. Such assays include, but are not limited to, radioimmunoassays, EIA, fluorescence, Western blot analysis and ELISAs. In one such embodiment, the
10 biological sample is contacted with antibodies of the present invention and a labeled second antibody is used to detect the presence of HCV to which the antibodies are bound.

15 Such assays may be, for example, direct where the labeled first antibody is immunoreactive with the antigen, such as, for example, a polypeptide on the surface of the virus; indirect where a labeled second antibody is reactive with the first antibody; a
20 competitive protocol such as would involve the addition of a labeled antigen; or sandwich where both labeled and unlabeled antibody are used, as well as other protocols well known and described in the art.

25 In one embodiment, an immunoassay method would utilize an antibody specific for HCV envelope determinants and would further comprise the steps of contacting a biological sample with the HCV-specific
30 antibody and then detecting the presence of HCV material in the test sample using one of the types of assay protocols as described above. Polypeptides and antibodies produced according to the present invention may also be supplied in the form of a kit, either
35 present in vials as purified material, or present in

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° compositions and suspended in suitable diluents as previously described.

5 In a preferred embodiment, such a diagnostic test kit for detection of HCV antigens in a test sample comprises in combination a series of containers, each container a reagent needed for such assay. Thus, one such container would contain a specific amount of HCV-specific antibody as already described, a second
10 container would contain a diluent for suspension of the sample to be tested, a third container would contain a positive control and an additional container would contain a negative control. An additional container could contain a blank.

15 For all prophylactic, therapeutic and diagnostic uses, the antibodies of the invention and other reagents, plus appropriate devices and accessories, may be provided in the form of a kit so as to facilitate ready availability and ease of use.

20 The present invention also relates to the use of nucleic acid sequences and polypeptides of the present invention to screen potential antiviral agents for antiviral activity against HCV. Such screening
25 methods are known by those of skill in the art. Generally, the antiviral agents are tested at a variety of concentrations, for their effect on preventing viral replication in cell culture systems which support viral replication, and then for an inhibition of infectivity
30 or of viral pathogenicity (and a low level of toxicity) in an animal model system.

In one embodiment, animal cells (especially human cells) transfected with the nucleic acid sequences
35 of the invention are cultured in vitro and the cells are

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° treated with a candidate antiviral agent (a chemical, peptide etc.) by adding the candidate agent to the medium. The treated cells are then exposed, possibly under transfecting or fusing conditions known in the art, to the nucleic acid sequences of the present invention. A sufficient period of time would then be allowed to pass for infection to occur, following which the presence or absence of viral replication would be determined versus untreated control cells by methods known to those of ordinary skill in the art. Such methods include, but are not limited to, the detection of viral antigens in the cell, for example, by immunofluorescence procedures well known in the art; the detection of viral polypeptides by Western blotting using antibodies specific therefor; the detection of newly transcribed viral RNA within the cells by RT-PCR; and the detection of the presence of live, infectious virus particles by injection of cell culture medium or cell lysates into healthy, susceptible animals, with subsequent exhibition of the signs and symptoms of HCV infection. A comparison of results obtained for control cells (treated only with nucleic acid sequence) with those obtained for treated cells (nucleic acid sequence and antiviral agent) would indicate, the degree, if any, of antiviral activity of the candidate antiviral agent. Of course, one of ordinary skill in the art would readily understand that such cells can be treated with the candidate antiviral agent either before or after exposure to the nucleic acid sequence of the present invention so as to determine what stage, or stages, of viral infection and replication said agent is effective against.

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In an alternative embodiment, viral enzyme such as NS3 protease, NS2-NS3 protease, NS3 helicase or NS5B RNA polymerase may be produced from a nucleic acid sequence of the invention and used to screen for inhibitors which may act as antiviral agents. The structural and nonstructural regions of the HCV genome, including nucleotide and amino acid locations, have been determined, for example, as depicted in Houghton, M. (1996), Fig. 1; and Major, M.E. et al. (1997), Table 2.

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Such above-mentioned protease inhibitors may take the form of chemical compounds or peptides which mimic the known cleavage sites of the protease and may be screened using methods known to those of skill in the art (Houghton, M. (1996) and Major, M.E. et al. (1997)). For example, a substrate may be employed which mimics the protease's natural substrate, but which provides a detectable signal (e.g. by fluorimetric or colorimetric methods) when cleaved. This substrate is then incubated with the protease and the candidate protease inhibitor under conditions of suitable pH, temperature etc. to detect protease activity. The proteolytic activities of the protease in the presence or absence of the candidate inhibitor are then determined.

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In yet another embodiment, a candidate antiviral agent (such as a protease inhibitor) may be directly assayed in vivo for antiviral activity by administering the candidate antiviral agent to a chimpanzee transfected with a nucleic acid sequence of the invention or infected with a virus of the invention and then measuring viral replication in vivo via methods such as RT-PCR. Of course, the chimpanzee may be treated with the candidate agent either before or after

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transfection with the infectious nucleic acid sequence or infected with a virus of the invention so as to determine what stage, or stages, of viral infection and replication the agent is effective against.

The invention also provides that the nucleic acid sequences, viruses and polypeptides of the invention may be supplied in the form of a kit, alone or in the form of a pharmaceutical composition.

All scientific publication and/or patents cited herein are specifically incorporated by reference. The following examples illustrate various aspects of the invention but are in no way intended to limit the scope thereof.

EXAMPLES

Materials and Methods

Source of HCV

An infectious plasma pool of HCV genotype 2a (HC-J6_{CH}) prepared from acute phase plasma of a chimpanzee experimentally inoculated with plasma from a Japanese patient infected with strain HC-J6 (Okamoto et al., 1991) was used for cloning. An infectious cDNA clone of HCV strain H77, genotype 1a was also used (pCV-H77C; Yanagi et al., 1997).

Amplification, cloning and sequence analysis

Viral RNA was extracted from 100 µl aliquots of the HC-J6_{CH} plasma pool with the TRIzol system (GIBCO/BRL) (Yanagi et al., 1997). Primers used in cDNA synthesis and PCR amplification were based on the genomic sequence of strain HC-J6 (Okamoto et al., 1991) and from the conserved region (3'X) of the 3' UTR of HCV genotype 2a (Tanaka et al., 1996) (Table 1). The RNA

- 30 -

was denatured at 65°C for 2 min, and cDNA was synthesized at 42°C for 1 hour with Superscript II reverse transcriptase (GIBCO/BRL) and specific reverse primers in 20 µl reaction volumes. The cDNA mixtures were treated with RNase H and RNase T1 (GIBCO/BRL) at 37°C for 20 min.

TABLE 1

Oligonucleotides used for amplification and cloning of strain HC-J6_{CH}, genotype 2a

Designation	Sequence (5' → 3') ^a
2427S-H77	ACTGGACACGGAGGTGGCCGCGTC
2426S-H77	TTGTTCTTGTCGGGTTAATGGCGC
2645R-H77	GGGTGTACTACACACATGAGTAAG
2832R-H77	AAGCGCCCTAACTGATGATG
H2751SII	CGTCATCGATA CCTCAGCGGGCATATGCACTGGACACGGA
H2786R	GTCCAGTGCATATGCCCCGCTGAGG
H2870R	CATGCACCAGCTGATATAGCGCTTGTAATATG
H7851S	TCCGTAGAGGAAGCTTGACGCTGACGCC
H9140S (M)	CAGAGGAGGCAGGTGCTATATGTGGCAAGTAC
H9173R (M)	GTACTTGCCACATATAGCAGCCCTGCCTCCTCTG
H9471R	CGTCTCTAGAC AGGAAATGGCTTAAGAGGCCGAGTGTTTACC
J6-H2556S	TTATGGATGCTCATCTTGTTGGGCCAGGCCGAAGCAGCTTTGGAGAACCTCGTAATACTCAATGC
356RF-J6H	AGGATTTGTGCTCATGGTGACGGTCTACGAG
1S-J6F ^b	TTTTTTTTTGGGCGCGCTAATACGACTCACTATAG ACCCGCCCCCTAATAGG
333S-J6	CCGTGCACCATGAGCACAAATCCTAAACCTC
753R-J6	GGATGTACCCCATGAGGTCCGCAAAG
2543S-J6F	GTTTGCGCTGCTTATGGATGCTCATCTTG
2787R-J6 (26)	GCGTCATAAGCATATGCCTGTTGGGG
3329R-J6	CCCTCAGCACTGGAGTACATCTG
5487-J6F	CGTCATGCATA CCCTAGGGCGGCTCTCATTGAAGAGGG
5518R-J6F	CGTCCCCTCTTCAATGAGAGCCGCTCTAGA
9251S-J6F	GCGGTGAAGACCAAGCTCAAACCTCACTC
9305R-J6F	AATCTAGA AGGCGCGCTTCCGGCAATGGAGTGAGTTTGAGC
9310R-J6F	CGTCTCTAGAG GATAAATCCAGGAGGCGCGCTTCCGGC
9399S-J6F	TACTTTTTGTAGGGGTAGGCCTTTTCC
9464-J6F	CGTCTCTAGAG TGTAGCTAATGTGTGCCGCTCTA
9470 (24) -J6	CTATGGAGTGTAGCTAATGTGTGC
J6-3' XR	CGTCTCTAGAC ATGATCTGCAGAGAGACCAGTTACGGCACTCTCTGFCAGTCATGCGGC
	TCACGGACCTTTCACAGCTAGCCGTGACTAGGGCTAAGATGGAGCCACC

^a HCV-specific sequences are shown in plain text, non HCV-specific sequences are shown in bold face, and cleavage sites used for cDNA cloning are underlined.

^b The core sequence of the T7 promotor is shown in italics.

The strategy used to amplify and clone the full-length HC-J6_{CH} sequence is shown in Fig. 1.

Nucleotide positions correspond to those of the 2a

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infectious clone (pJ6CF) that is described herein. The 5' end of HC-J6_{CH} (nts. 17-297, excluding primer sequences) was amplified from 2 µl of cDNA synthesized with primer a-2 (Yanagi et al., 1996). PCR was performed with *AmpliTag Gold* DNA polymerase (Perkin-Elmer) as described previously (Yanagi et al., 1996) using primers 1S-J6F and a-2. After purification, the amplified products were cloned into pGEM-T Easy vector (Promega) using standard procedures and 5 clones (pJ6-5'UTR) were sequenced.

The 3' end of HC-J6_{CH} was amplified in 3 overlapping pieces. RT-PCR of a short fragment of NS5B (nts. 9279-9439) was performed with primers 9251S-J6F and 9464R-J6F as described above. The PCR products were cloned into pGEM-T Easy vector and sequence analysis was performed from 5 pJ6-3'F clones. A second region spanning from NS5B to the conserved region of the 3' UTR (nts. 9376-9629) was amplified in RT-nested PCR (external primers H9261F and H3'X58R, internal primers H9282F and H3'X45R) (Yanagi et al., 1997). The amplified products were cloned into pGEM-9zf(-) by using *HindIII* and *XbaI* sites and 14 pJ6-3'VR clones were sequenced. The third fragment, which included the 3' terminal sequence was amplified with primers 9399S-J6F and J6-3'XR from one of the pJ6-3'VR clones, and cloned into one of the pJ6-3'F clones by using *StuI* and *XbaI* sites (pJ6-3'X).

The ORF of HCV HC-J6_{CH} was amplified by long RT-PCR in 3 overlapping pieces. The amplification was performed on 2 µl of the cDNA mixtures with the Advantage cDNA polymerase mix (Clontech) (Yanagi et al., 1997). The J6S fragment (nts. 86-2761) was amplified

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with primers a-1 (Yanagi et al., 1996) and J6-2787R from
cDNA synthesized with primer J6-3329R. A single PCR
round was performed in a Robocycler thermal cycler
(Stratagene), and consisted of denaturation at 99°C for
5 35 sec, annealing at 67°C for 30 sec and elongation at
68°C for 4 min 30 sec during the first 5 cycles, 5 min
during the next 10 cycles, 5 min 30 sec during the
following 10 cycles and 6 min during the last 10 cycles.
10 The J6B fragment (nts. 2573-5488) was amplified with
primers 2543S-J6F and 5518R-J6F from cDNA synthesized
with primer 5518R-J6F. Finally, the J6A fragment (nts.
5515-9282) was amplified with primers 5487S-J6F and
9310R-J6F from cDNA synthesized with primer
15 9470R(24)-J6F. PCR amplifications of fragments J6B and
J6A consisted of denaturation at 99°C for 35 sec,
annealing at 67°C for 30 sec and elongation at 68°C for 6
min during the first 5 cycles, 7 min during the next 10
cycles, 8 min during the following 10 cycles and 9 min
20 during the last 10 cycles.

After purification of the long PCR products
with QIAquick PCR purification kit (QIAGEN), A-tailing
reactions were performed with *AmpliTag* DNA polymerase
25 (Perkin Elmer) at 72 °C for 1 hour. The gel-purified
A-tailed PCR products were cloned into pCR2.1 vector
(Invitrogen) or pGEM-T Easy vector (Promega). DH5-alpha
competent cells (GIBCO BRL) were transformed and
30 selected on LB agar plates containing 100 µg/ml
ampicillin (SIGMA) and amplified in LB liquid cultures
at 30°C for 18 - 20 hrs (Yanagi et al., 1997). Midiprep
was performed using Wizard *Plus* Midipreps DNA
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Purification System (Promega). Multiple clones of the J6S, J6A and the J6B fragments were sequenced.

The consensus sequence of strain HC-J6_{CH} (nts. 17-9629) was determined by direct sequencing of PCR products (nts. 297-3004 and nts. 4893-5762) and by sequence analysis of the TA clones (nts. 17-5488 and nts. 5515-9629) (Fig. 1). Both strands of DNA were sequenced in all cases. Analyses of genomic sequences, including multiple sequence alignments and tree analyses, were performed with GeneWorks (Oxford Molecular Group) (Bukh *et al.*, 1995).

Construction of chimeric cDNA clones of genotypes 1a & 2a

Four full-length intertypic chimeric cDNA clones were constructed (Figs. 4, 5A, 5B). In each clone the C, E1 and E2 genes encoded the consensus amino acid sequence of HC-J6_{CH}. The p7 protein was encoded either by the HC-J6_{CH} or pCV-H77C consensus sequence, and the NS proteins were all encoded by pCV-H77C genes. To engineer these cDNA clones, an *Nde*I site from pCV-H77C was first eliminated by a silent substitution (C to T) at position 9158. In brief, two fragments were amplified from pCV-H77C with primers H7851S and H9173R(M) and with primers H9140S(M) and H9417R (Table 3), gel-purified and used for fusion PCR with primers H7851S and H9417R. The fusion PCR products were cloned into pCV-H77C by using *Hind*III and *Afl*III sites. A new artificial *Nde*I site was introduced by a silent substitution (C to T) at position 2765. PCR products, which were amplified from pCV-H77C with primer H2751SII containing artificial *Cla*I and *Nde*I sites and primer H2870R, were cloned into the modified pCV-H77C by using

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° *Cla*I and *Eco*47III sites. The final construct (pH77CV) was used as a cassette vector to construct the intertypic chimeric HCV cDNA clones.

5 The four chimeric cDNA clones were constructed as follows. pH77CV-J6S (nucleotide sequence shown in SEQ ID No:3 and amino acid sequence shown in SEQ ID No:4): The *Age*I/*Bsm*I fragment of clone J6S2 and the *Bsm*I/*Nde*I fragment of clone J6S1, were cloned into pH77CV by using *Age*I and *Nde*I sites; pH77 (p7)CV-J6S
10 (nucleotide sequence shown in SEQ ID No:5 and amino acid sequence shown in SEQ ID No:6): A fragment of pH77CV-J6S was replaced with a fragment amplified from pCV-H77C with primers J6-H2556S and H2786R by using *Bsa*BI and *Nde*I sites; J6S (nucleotide sequence shown in SEQ ID
15 No:7 and amino acid sequence shown in SEQ ID No:8): A fragment amplified from pH77pCV-H77C with primers a-1 and 356RF-J6H77 and another fragment amplified from pH77CV-J6S with primers 333S-J6 and 753R-J6 were
20 gel-purified and a fusion-PCR was performed with primers a-1 and 753R-J6. The *Age*I/*Cla*I fragment of the subcloned fusion PCR products and the *Cla*I/*Nde*I fragment of pH77CV-J6S were cloned into pH77CV-J6S by using *Age*I
25 and *Nde*I sites; pH77(p7)-J6S (nucleotide sequence shown in SEQ ID No:9 and amino acid sequence shown in SEQ ID No:10): The *Age*I/*Cla*I fragment of J6S and the *Cla*I/*Nde*I fragment of (p7)CV-J6S were cloned into pH77(p7)CV-J6S
30 by using *Age*I and *Nde*I sites.

Each intertypic chimeric cDNA clone was retransformed to select a single clone, and large-scale preparation of plasmid DNA was performed with a QIAGEN plasmid Maxi kit as described previously (Yanagi et al.,
35 1997). Each of the four cDNA clones was completely

- 35 -

sequenced before inoculation. Each clone was genetically stable since the digestion pattern was as expected following retransformation and the complete sequence was the expected one.

5 Construction of full-length cDNA clone HC-J6_{CH}

An overview of the full-length HC-J6_{CH} clone is presented in Fig. 1. In the final construct pJ6CF, which encodes the consensus polyprotein of HC-J6_{CH}, an *Xba*I site was eliminated by a silent substitution (A to G) at position 5494. Digested fragments containing the consensus sequence were purified from the appropriate subclones and ligated using the sites indicated. The full-length cDNA clone (pJ6CF) was retransformed to select a single clone, and large-scale preparation of plasmid DNA followed by the complete sequence analysis was performed. Clone pJ6CF was genetically stable.

20 Intrahepatic transfection of chimpanzee with transcribed RNA

In duplicate 100 µl reactions, RNA was transcribed *in vitro* with T7 RNA polymerase (Promega) from 10 µg of template plasmid linearized with *Xba*I (Promega) as described previously (Yanagi et al., 1997). The integrity of the RNA was checked by electrophoresis through agarose gel stained with ethidium bromide (Yanagi et al., 1997). Each transcription mixture was diluted with 400 µl of ice-cold phosphate-buffered saline without calcium or magnesium and then immediately frozen on dry ice and stored at -80°C. Within 24 hours, both transcription mixtures were injected into the same chimpanzee by percutaneous intrahepatic injection guided by ultrasound (Yanagi et al., 1998, 1999). If the

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chimpanzee did not become infected, the same transfection was repeated once. After two negative results, the next clone was inoculated into the same chimpanzee following the same protocol. Injections were performed at weeks 0 and 2 with pH77CV-J6S, at weeks 5 and 8 with pH77(p7)CV-J6S, at weeks 14 and 16 with pH77-J6S, at weeks 19 and 23 with pH77(p7)-J6S, at week 28 with pJ6CF, and finally at week 34 with pCV-H77C. The chimpanzee was maintained under conditions that met or exceeded all requirements for its use in an approved facility.

Serum samples were collected weekly from the chimpanzee and monitored for liver enzyme levels by standard procedures, anti-HCV antibodies by the second-generation ELISA (Abbott) and HCV RNA by a sensitive RT-nested PCR assay with *AmpliTaq Gold* DNA polymerase using primers from the 5' UTR (Yanagi et al., 1996). Samples were scored as negative for HCV RNA if two independent tests on 100 µl of serum were negative. The genome equivalent (GE) titer of HCV in positive samples was determined by RT-nested PCR on 10-fold serial dilutions of the extracted RNA (Bukh et al., 1998). The consensus sequence of the complete ORF from the chimpanzee infected with RNA transcripts of pJ6CF was determined by direct sequencing of overlapping PCR products obtained by long RT-nested PCR as previously described (Yanagi et al., 1997) with HC-J6 specific primers. After the intrahepatic transfection with RNA transcripts of pCV-H77C, we performed H77(genotype 1a)-specific RT-nested PCR with primers 2427S-H77 and 2832R-H77 for the 1st round and with primers 2462S-H77 and 2645R-H77 for the 2nd round (Table 3). The

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° sensitivity of this assay was equivalent to that of the assay using 5' UTR primers when testing serum containing only H77, genotype 1a. The genome titer of genotype 1a was determined by using this specific RT- nested PCR on
5 10-fold serial dilutions of the extracted RNA.

EXAMPLE 1

Sequence analysis of HCV strain HC-J6_{CH}

10 As minor deviations from the consensus amino acid sequence were found previously to render full-length HCV cDNA clones noninfectious (Yanagi et al., 1997, 1998), the consensus sequence of the cloning source of genotype 2a (strain HC-J6_{CH}) was determined
15 prior to constructing any full-length clones. In brief, a plasma pool containing strain HC-J6_{CH} was prepared from acute phase plasmapheresis units collected from a chimpanzee experimentally infected with HC-J6 (Okamoto et al., 1991). The HCV genome titer of this pool was
20 10^{5.4} genome equivalents (GE)/ml (Quantiplex HCV RNA bDNA 2.0, Chiron) and the infectivity titer was 10⁴ chimpanzee infectious doses/ml.

25 The consensus sequence of the 5' UTR of HC-J6_{CH} (nts. 17-340) was deduced from 5 clones containing nts. 17-297 and 8 clones containing nts. 86-340. The 5' UTR of the various clones was highly conserved, but the consensus sequence of HC-J6_{CH} differed by 2 nucleotides from that published previously for HC-J6 (Okamoto et al., 1991: C to T at position 36 and T to C at position
30 222).

35 The consensus sequence of 14 clones of the 3' UTR of HC-J6_{CH} indicated that the 39 nucleotide long variable region was highly conserved in this strain and

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° was identical to that previously published for HC-J6 (Okamoto *et al.*, 1991). The polypyrimidine tract varied greatly in length (84-164 nucleotides), and contained some conserved A residues. In the conserved region, the proximal 16 nucleotides were identical to those previously published for isolates of different HCV genotypes (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1996; Yamada *et al.*, 1996). The remaining 82 nucleotides of the conserved region were determined for other genotype 2a strains (Tanaka *et al.*, 1996) but not for HC-J6 or HC-J6_{CH}.

The ORF of HC-J6_{CH} was amplified in 3 fragments by RT-PCR (Fig. 1). Eight clones of the J6S fragment (nts. 86-2761), 6 clones of the J6B fragment (nts. 2573-5488) and 6 clones of the J6A fragment (nts. 5515-9298) were sequenced. PCR fragments containing nts. 5489-5514 were sequenced directly. A quasispecies was found at 243 nucleotide (2.7%) and 69 amino acid (2.3%) positions, scattered throughout the 9099 nts (3033 aa) of the ORF. However, the majority, 231 nucleotide substitutions, were detected only once and 71.6 % of these represented silent mutations. The 12 remaining nucleotide substitutions were each restricted to 2 clones and only 4 of these resulted in amino acid changes. The nucleotide difference among the J6S clones ranged from 0.1 - 1.3%, among the J6B clones it ranged from 0.1 - 0.3%, and it ranged from 0.2 - 4.0% among the J6A clones (Fig. 2). Three of 8 J6S clones, 4 of 6 J6B clones, and all 6 J6A clones had defective polyproteins due to nucleotide deletions, insertions or substitutions.

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- 39 -

° The sequences of clones of strain HC-J6_{CH} were relatively homogeneous. This was highlighted by the high degree of conservation among clones of the HVR1 (Fig. 3), a region frequently used to study the quasispecies of HCV (Bukh et al., 1995). An exception was the sequence of clone J6A1, which differed by about 4% from the other clones of this region (Fig. 2). Importantly, the consensus sequence of strain HC-J6_{CH} (nts. 17-9629) could be determined with no ambiguity at the nucleotide or deduced amino acid level. The difference between the consensus ORF sequence of HC-J6_{CH} from the experimentally infected chimpanzee and that of HC-J6 of the inoculum (Okamoto et al., 1991) was 4.1 % and 2.2 % at the nucleotide and deduced amino acid levels, respectively (Fig. 2, Table 2). Moreover, we found that 12 (44.4%) of the 27 amino acids constituting HVR1 differed between HC-J6_{CH} and HC-J6 (Fig. 3). Such diversities are greater than the < 2 % generally considered to comprise a quasispecies. In fact, these differences are equivalent to those found between the two prototype strains of HCV genotype 1a [strains HCV-1 (Choo et al., 1991) and H77 (Yanagi et al., 1997)]. These results indicated that HC-J6_{CH}, which represented the major species in the experimentally infected chimpanzee, was a minor species in the original inoculum.

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TABLE 2

Percent difference of nucleotide and predicted amino acid sequences between strain HC-J6 (Okamoto *et al.*, 1991) and strain HC-J6_{CH} from acute phase plasma pool of a chimpanzee inoculated with HC-J6

Genome Region	nt.position ^a	% nt. difference	% a.a. difference
ORF	341-9439	4.1 (373/9099) ^b	2.2 (66/3033) ^b
5' UTR	17-340	0.6 (2/324)	
Core	341-913	0.5 (3/573)	0 (0/191)
E1	914-1489	4.3 (25/576)	2.1 (4/192)
HVR1	1490-1570	24.7 (20/81)	44.4 (12/27)
E2-HVR1	1571-2590	3.9 (40/1020)	3.2 (11/340)
p7	2591-2779	3.7 (7/189)	3.2 (2/63)
NS2	2780-3430	4.0 (26/651)	2.8 (6/217)
NS3	3431-5323	4.0 (76/1893)	0.8 (5/631)
NS4A	5324-5485	4.3 (7/162)	1.9 (1/54)
NS4B	5486-6268	3.7 (29/783)	0.4 (1/261)
NS5A	6269-7666	5.4 (75/1398)	3.4 (16/466)
NS5B	7667-9439	3.7 (65/1773)	1.4 (8/591)
3' UTR	9440-9481	0 (0/42)	

^a The nucleotide positions correspond to those of the infectious full-length genotype 2a clone (pJ6CF).

^b The numbers in parenthesis indicate the nucleotide or amino acid differences for each region.

Example 2

Chimeric molecular clones

As chimeric flaviviruses with substituted structural genes have been useful in defining the biological function of viral sequences or proteins, in analyzing immune responses and in generating attenuated vaccine candidates (Bray and Lai, 1991; Chambers *et al.*, 1999; Pletnev *et al.*, 1992, 1993, 1998). The consensus sequence of the 2a structural genes and surrounding region was substituted for that of the infectious 1a cDNA clone. In the genotype 1a backbone, two silent mutations were introduced for cloning purposes [at positions 2765 (p7) and 9158 (NS5B) of pCV-H77C] (Fig. 4). The complete sequence of each chimera was verified. Infectivity of RNA transcripts from four different

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intertypic chimeric clones (Figs. 4, 5A, 5B) was evaluated by consecutive intrahepatic transfections of a chimpanzee. Clones were considered not to be viable if viral RNA was not detected in the serum within two weeks of the repeat transfection. All chimeric clones contained the C, E1 and E2 genes of genotype 2a. The two chimeric clones tested initially differed from each other in that one had the p7 gene of 2a (pH77CV-J6S) and the other [pH77(p7)CV-J6S] the p7 gene of 1a. They differed from the two other clones in that the 186 nucleotides of the 5' UTR just upstream of the initiation codon were from the 2a genotype. Since neither clone containing the chimeric 5' UTR was infectious, the chimeric 5' UTR was replaced with the consensus genotype 1a 5' UTR to generate the two p7 varieties [pH77-J6S and pH77(p7)-J6S]. After consecutive transfection of the four clones, no HCV RNA, anti-HCV or ALT elevation was detected in the chimpanzee during 28 weeks of follow-up, suggesting that RNA transcripts from these intertypic chimeric clones were not viable *in vivo*.

This finding that the intertypic clones between genotypes 1a and 2a were not viable was surprising since flavivirus chimeras containing the structural region of dengue virus type 1 or 2 or of tick-borne encephalitis virus and the nonstructural region of an infectious dengue type 4 virus were viable (Bray and Lai, 1991; Pletnev et al., 1992, 1993). While considerable sequence variation exists between the infectious genotype 1a and 2a clones of HCV (Table 3), these viruses exhibit a higher degree of genetic heterogeneity than do the major genotypes of HCV. For other flaviviruses, however, it was possible to obtain

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infectious chimeric clones only if the capsid region was derived from the backbone cDNA clone (Chambers *et al.*, 1999; Pletnev and Men, 1998).

TABLE 3

Percent difference of the amino acid sequences between the infectious clone of genotype 1a (pCV-H77C; Yanagi *et al.*, 1997) and the infectious clone of genotype 2a (pJ6CF) of hepatitis C virus

Genome Region ^a	% difference
Polyprotein	27.9 (839/3007) ^b
Core	8.9 (17/191)
E1	37.0 (71/192)
HVR1	59.3 (16/27)
E2-HVR1	27.1 (91/336)
p7	38.1 (24/63)
NS2	41.9 (91/217)
NS3	19.2 (121/631)
NS4A	33.3 (18/54)
NS4B	26.8 (70/261)
NS5A	38.5 (171/444)
NS5B	25.2 (149/591)

^a Genome regions defined as in Table 1.

^b The numbers in parenthesis indicate the amino acid differences for each region. Positions with deletions or insertions in E2 (4 aa positions) and NS5A (26 aa positions) were not considered.

Trivial explanations may account for the lack of viability of these intertypic chimeras. First, the two silent mutations introduced in the genotype 1a backbone (one in p7 and one in NS5B) for cloning purposes could potentially eliminate infectivity. This is, however, very unlikely since mutations at these positions exist among field isolates of HCV including strain HC-J6_{CH} (Bukh *et al.*, 1998). Also, it is noteworthy that the three previously published infectious clones of strain H77 had numerous silent nucleotide differences (Hong *et al.*, 1999; Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997). Second, signal peptidases might not cleave the chimeric E2/p7 or p7/NS2

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junction. This seems unlikely, however, since
eukaryotic signal peptidases typically recognize the
amino acid sequences upstream of the cleavage site [the
(-3, -1) rule] (Nielsen et al., 1997) and the amino
acids at these two sites are conserved between genotypes
1a and 2a (Fig. 5B). Finally, the E2/p7 and/or p7/NS2
gene junctions could differ between genotypes 1a and 2a.
The junctions determined for genotypes 1a and 1b were
used (Lin et al., 1994; Mizushima et al., 1994; Selby et
al., 1994) because those for genotype 2a have not been
identified. In the latter two cases, further analyses
of genotype 2a should eventually provide sufficient data
to overcome such potential problems and it would most
likely be possible to construct a viable chimera.

More complicated explanations for the lack of
viability of the chimeras might be required if critical
genotype-specific interactions occur as regards the
structural proteins, the nonstructural proteins and the
genomic RNA. For instance, one cannot rule out that the
chimeras were not viable because the IRES function was
compromised. In *in vitro* studies the IRES activity
depended on RNA sequences not only in the 5' UTR but
also extending 3' of the translation initiation site
(Hahm et al., 1998; Lemon and Honda, 1997; Reynolds et
al., 1995). Although the 3' border of the HCV IRES is
still controversial it is believed to involve at most
the first 39 nts of the core gene (Lemon and Honda,
1997). The 5' UTR of the intertypic chimeras was either
a chimera of genotype 1a and 2a sequences or the entire
5' UTR was derived from the 1a clone (Figs. 4, 5A).
Importantly, the 5' end of core is conserved among
genotypes 1a and 2a (Fig. 5A). Thus, the predicted

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° IRES-like secondary structure is maintained in these chimeras, suggesting that the IRES activity most likely was maintained.

Possible interactions between the structural proteins and the nonstructural proteins and/or the genomic RNA, which involve RNA packaging, replication or translation are conceivable. In poliovirus, which is another positive-sense RNA virus, functional coupling of RNA packaging to RNA replication and of RNA replication to translation have been suggested (Novak and Kirkegaard, 1994 ; Nugent et al., 1999). Similar to other viruses of the *Flaviviridae* family, a membrane-associated replicase complex is thought to initiate replication at the 3' end of HCV and to synthesize a complementary negative-strand RNA (Rice, 1996). The putative cis-acting elements at the 5' and 3' termini which are believed to be important for viral genome replication (Rice 1996; Frolov et al., 1998) should be maintained in the intertypic HCV chimeras at least in the two constructs with the authentic 1a 5'UTR. However, it is conceivable that the viral packaging system was interrupted (Frolov et al., 1998). Studies using a Kunjin flavivirus replicon system and providing the structural proteins *in trans* suggested that the essential encapsidation signals did not reside in the structural region of the genome (Khromykh et al., 1997, 1998). The location of the packaging signals of HCV is not known. However, if the structural proteins encapsidate viral RNA via genotype-specific sequences outside of the structural region, the chimeras would be unable to package the RNA and it might be extremely

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- ° difficult to construct viable chimeras between highly divergent strains.

Example 3

5 A consensus molecular clone of
 genotype 2a is infectious in vivo

 In order to prove that the genotype 2a portion used in the 4 intertypic chimeric cDNA clones indeed represented the infectious sequence, a consensus full-length cDNA clone of HC-J6_{CH} (pJ6CF) was constructed. The core sequence of the T7 promoter, a 5' guanosine residue and the full-length sequence of HC-J6_{CH} (9711 nts) were cloned into pGEM-9Zf vector using NotI/XbaI sites. Within the HCV sequence there were no deduced amino acid differences and only 4 nucleotide differences (at nucleotide positions 1822, 5494, 9247 and 9289) from the consensus sequence of HC-J6_{CH} as determined in the present study. The silent mutation at position 1822 was within the structural region and so was also present in the four intertypic chimeras. The 5' terminal 16 nts and the 3' terminal 82 nts were deduced from previously published HCV genotype 2a sequences (Okamoto et al., 1991, Tanaka et al., 1996). The full-length cDNA clone of genotype 2a contained a 5' UTR of 340 nts, an ORF of 9099 nts encoding 3033 amino acids and a 3' UTR consisting of a variable region of 39 nts followed by a 132 nucleotide-long polypyrimidine tract interrupted with 3 A residues and the 3' terminal conserved region of 98 nts.

 RNA transcripts from pJ6CF were injected into the same chimpanzee used for injection of the 4 intertypic chimeras. The chimpanzee became infected at

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the first attempt with an HCV titer of 10^2 GE/ml at week 1 post inoculation (p.i.), and 10^3 - 10^4 GE/ml during weeks 2 to 6 p.i. The consensus sequence of PCR products of the complete ORF, amplified from serum obtained during week 5 p.i., was identical to the sequence of pJ6CF and there was no evidence of a quasispecies. Since RNA transcripts of this infectious genotype 2a clone were infectious *in vivo*, and it shared an exact sequence with the non-infectious intertypic chimeric clones, their failure to replicate must have been the result of incompatibilities between the genotype 1a and 2a sequences.

To confirm that the chimpanzee used was susceptible also to infection by genotype 1a, which comprised most of the intertypic chimeras, the chimpanzee was subsequently inoculated with RNA transcripts from the infectious genotype 1a clone (pCV-H77C). Serum samples were tested in an H77-specific RT-PCR assay to identify super-infection with genotype 1a. At week 1 p.i. the total HCV genome titer was 10^4 GE/ml and the H77-specific (1a) genome titer was 10^2 GE/ml. The H77-specific genome titer increased to 10^3 GE/ml at week 2 p.i., and reached 10^4 GE/ml during weeks 3-6 p.i. The consensus sequence of PCR products amplified with H77-specific primers at weeks 1-6 p.i. were found to be identical to that of pCV-H77C. However, the direct sequences of PCR products amplified with the 5' UTR primers at weeks 1-2 after inoculation of pCV-H77C were identical to that of pJ6CF indicating that the 2a genotype was still present and represented the majority species. These experiments confirmed that the inability of the intertypic 1a, 2a

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° cDNA clones to infect the chimpanzee was not the result of protective immune responses in the chimpanzee but represented deficiencies intrinsic to the chimeras.

Discussion

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The published infectious cDNA clones of HCV represent the two most important subtypes of genotype 1 (Hong et al., 1999; Kolykhalov et al., 1997; Yanagi et al., 1997, 1998). However, 5 more major genotypes of HCV are recognized. In the above Examples, the infectivity of a cDNA clone of a second major HCV genotype was demonstrated. As in previous studies, the infectivity of RNA transcripts was demonstrated *in vivo* by intrahepatic transfection of a chimpanzee. This new infectious clone (pJ6CF) encodes the consensus polyprotein of HCV strain HC-J6_{CH}, genotype 2a. Its encoded polyprotein differs from those of the infectious clones of genotypes 1a and 1b by approximately 30% (Table 2). Genotype 2 strains, in particular subtypes 2a and 2b, have a worldwide distribution and important differences between genotypes 1 and 2 with respect to pathogenesis and treatment were indicated in previous studies. The availability of an infectious clone representing a second major genotype of HCV should permit new ways of studying the molecular biology and immunopathology of this important and genetically quite different human pathogen.

The 5' and 3' UTRs of HCV are believed to be critical for viral replication, translation and viral packaging (Rice, 1996). The 5' 203 terminal nucleotides and the 3' 101 terminal nucleotides of the published infectious clones of genotypes 1a and 1b were identical.

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However, the sequences of UTRs of the genotype 2a clone differ from those of the genotype 1 clones. Overall, the 5' UTR of the genotype 2a clone has 17 nt differences and a single nucleotide deletion compared with the infectious clones of genotype 1a (Fig. 5A). Five of these differences and the deletion are within the first 30 nucleotides, whereas the remainder are found within the predicted IRES structure. Differences also exist between the 3' UTR of the genotype 2a clone and the clones of genotype 1a (Fig. 5B). The sequences of the variable region are very different. Recent study has shown this region is not critical for infectivity *in vivo* (Yanagi *et al.*, 1999). Within the regions which are critical for infectivity *in vivo* (Yanagi *et al.*, 1999), the 132 nucleotide-long polypyrimidine tract of the genotype 2a clone has 3 unique A residues interspersed and the 3' terminal conserved region of 98 nts has 4 nt differences within the 3' terminal stable stem-loop structure (Fig. 5B) (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1996). Since the 2a clone was infectious these sequence differences are apparently real and are compatible with infectivity. Further studies are required to determine whether these represent critical genotype-specific sequences.

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